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Toll-like receptor 4-mediated growth of endometriosis by human heat-shock protein 70

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BACKGROUND: We investigated the role of human heat-shock protein 70 (Hsp70) in Toll-like receptor 4 (TLR4)-mediated growth of endometriosis. **METHODS:** TLR4 expression was examined in macrophages (M ϕ) isolated in primary culture from the peritoneal fluid of women with and without endometriosis. The production of a number of macromolecules by non-treated M ϕ , Hsp70-treated M ϕ and after treatment with anti-TLR4 antibody was examined by enzyme linked immunosorbent assay (ELISA). The single and combined effects of Hsp70 and lipopolysaccharide (LPS) on the growth of endometrial stromal cells were analyzed by 5-bromo-2-deoxyuridine (BrdU) incorporation study. Hsp70 levels in eutopic and ectopic endometria were measured by ELISA. **RESULTS:** TLR4 was detected in isolated M ϕ at protein and gene level. Hsp70 (10 μ g/ml) significantly stimulated the production of hepatocyte growth factor, vascular endothelial cell growth factor, interleukin-6 and tumor necrosis factor alpha by M ϕ derived from women with endometriosis compared with M ϕ derived from women with no endometriosis ($P < 0.05$ for each). This effect of Hsp70 was abrogated after pretreatment of M ϕ with anti-TLR4 antibody. BrdU incorporation indicated that Hsp70 significantly enhanced the growth of endometrial stromal cells (~50% increase) from women with endometriosis compared to non-treated cells. A synergistic effect on cell proliferation was observed between exogenous Hsp70 and LPS and this was significantly suppressed by pretreatment of cells with anti-TLR4 antibody ($P < 0.05$). Tissue levels of Hsp70 were significantly higher in the eutopic endometria ($P < 0.05$) and opaque red lesions ($P < 0.01$) derived from women with endometriosis than from other peritoneal lesions or from women with no endometriosis. **CONCLUSIONS:** A prominent stress reaction was observed in blood-filled opaque red peritoneal lesions. Human Hsp70 induces pelvic inflammation and may be involved in TLR4-mediated growth of endometrial cells derived from women with endometriosis.

Keywords: cell growth; endometriosis; TLR4; Hsp70; LPS; macrophages

Introduction

Endometriosis induces a variable amount of inflammatory reaction in the pelvic environment depending on the stage and morphology of the disease (Halme *et al.*, 1987; Harada *et al.*, 2001; Lebovic *et al.*, 2001; Khan *et al.*, 2003; Wira *et al.*, 2005). The inflammatory reaction associated with endometriosis was demonstrated both *in vitro* and *in vivo* by the infiltration of immune cells and the presence of a number of primary and secondary inflammatory mediators in tissue and body fluids (Halme *et al.*, 1988, 1989; Keenan *et al.*, 1994, 1995; Osuga *et al.*, 1999; Mahnke *et al.*, 2000; Khan *et al.*, 2002a,b, 2004a, 2005a, 2007). The primary inflammatory mediators derived from Gram-negative and -positive microbes can elicit an immune response in the pelvic environment through pattern-recognition receptors, which belong to the

family of Toll-like receptors (TLRs) (Akira and Takeda, 2004; Takeda and Akira, 2005; Khan *et al.*, 2007). Other secondary inflammatory mediators that are produced in response to primary inflammatory mediators interact with their respective receptors in immune cells or endometrial cells and may be involved in the growth of endometriosis either alone or in combination (Halme *et al.*, 1987; McLaren *et al.*, 1996; Harada *et al.*, 2001; Khan *et al.*, 2005a,b,c). There is a possibility that, in addition to the pelvic inflammation, endometriosis may also produce a stress reaction and release endogenous heat-shock proteins (Hsps) in the pelvic environment as a result of tissue damage, tissue invasion and by inflammatory reaction itself. However, studies are limited regarding immune cell-mediated regulation of pelvic endometriosis by human Hsp, their mechanism of action and levels in the pelvic environment.

A wide variety of stressful stimuli, such as heat shock, ultraviolet radiation, viral or bacterial infections, internal physical stress, chemical stress and pelvic inflammation, induce an increase in the intracellular synthesis of Hsps (Zugel and Kaufmann, 1999; Asea *et al.*, 2000, 2002). Mammalian Hsp60, Hsp70 and Hsp90 have been implicated in a variety of autoimmune and inflammatory conditions (Wallin *et al.*, 2002). The so-called 'danger theory' states that antigen presenting cells can be activated by endogenous substances released by damaged or stressful tissues (Matzinger, 1998). Members of the Hsp family are candidate molecules that potentially signal tissue damage or cellular stress to the immune system.

Human Hsps (Hsp60, Hsp70 and Hsp90) are reported to be produced by macrophages (M ϕ), vascular endothelial cells, smooth muscle cells, endometrial cells and other dendritic cells (Wallin *et al.*, 2002). In a manner similar to the recognition of lipopolysaccharide (LPS), recognition of Hsp60 and Hsp70 seems to be mediated by a complex of TLR4 and MD-2 (Kol *et al.*, 1999; Wallin *et al.*, 2002). Since the biological potentiality of human Hsp70 is stronger than that of either Hsp60 or Hsp90 (Wallin *et al.*, 2002), we report here TLR4 expression in M ϕ derived from the peritoneal fluid (PF) of women with and without endometriosis and TLR4-mediated growth of pelvic endometriosis in response to human Hsp70. We also examined the pattern of stress reaction by measuring endogenous Hsp70 concentration in the eutopic endometria and different peritoneal lesions of women with pelvic endometriosis.

Materials and Methods

Subjects

A total of 25 women between 20 and 38 years of age undergoing laparoscopy for pelvic pain, dysmenorrhea and/or infertility were recruited in this study. Among them, 12 women had endometriosis of Stages I–II and the remaining 13 women had endometriosis of Stages III–IV at the time of diagnostic laparoscopy. The control group consisted of 12 fertile women between 21 and 36 years of age without any evidence of pelvic or ovarian endometriosis and operated for dermoid cysts by laparoscopy. The staging and the morphological distribution of peritoneal lesions were based on the revised classification of The American Society of Reproductive Medicine (r-ASRM) (ASRM, 1997). Neither the study group nor the endometriosis free group had been on hormonal medication in 3 months prior to the surgical procedure. All control women and women with endometriosis had regular menstrual cycles (28–32 days). The phase of the menstrual cycle was determined by histological dating of eutopic endometrium samples taken simultaneously with the PF samples. Menstrual dating was carried out by an independent pathologist. All induced menstrual cycles were excluded from the current study. The distribution of patients in different menstrual cycles is as follows: control women, proliferative phase, $n = 4$; secretory phase, $n = 8$; endometriosis women, proliferative phase, $n = 10$; secretory phase, $n = 15$.

Peritoneal lesions of endometriosis were diagnosed by their macroscopic appearances according to the published criteria (Jansen and Russel, 1996) and categorized as red, black and white lesions as proposed in the latest revision of the ASRM classification (ASRM, 1997). As we described recently (Khan *et al.*, 2004b), the distribution of peritoneal lesions according to color appearance for our current study was done as follows: total red lesions ($n = 20$) [blood-filled

opaque red lesions ($n = 8$) and non-opaque transparent and/or translucent red lesions ($n = 12$]; black lesions ($n = 21$), and white lesions ($n = 11$). Biopsy specimens from each of these peritoneal lesions were collected for subsequent experimental analysis. The details of physical collection of tissue biopsies and the PF were reported elsewhere (Khan *et al.*, 2002b, 2004a,b, 2006).

All biopsy specimens and the PF were collected in accordance with the guidelines of the Declaration of Helsinki and with the approval by the Nagasaki University Institutional Review Board. An informed consent was obtained from all women.

Isolation of the M ϕ from the PF

The PF was obtained from all women with or without endometriosis with the use of laparoscopy. The M ϕ were isolated in primary culture from the PF of six women with and without endometriosis. The detailed procedure of M ϕ isolation in primary culture was described previously (Khan *et al.*, 2005a,b). The M ϕ were allowed to adhere to the culture plate for 2 h, after which the non-adherent cells were removed by washing the plates three times with RPMI medium. The adherent cells remaining on the plates were more than 95% M ϕ as estimated by their morphology and by immunocytochemical staining using CD68 (KP1), a mouse monoclonal antibody from Dako, Denmark. An aliquot of M ϕ was plated in four-well chamber slides (Nunc, Naperville, IL, USA) for immunostaining and the rest were used for culture. The detailed procedure of immunocytochemical staining is described elsewhere (Rana *et al.*, 1996; Khan *et al.*, 2003, 2004a). Non-immune mouse immunoglobulin (Ig) G1 antibody in 1:50 dilution was used as a negative control. A counter staining of M ϕ with hematoxylin–eosin was also performed and we did not find any contaminating cells, such as gland cells or stromal cells in isolated M ϕ (data not shown).

Isolation of stromal cells in primary culture

Stromal cells were collected from the biopsy specimens of the eutopic endometria derived from six women with and without endometriosis. The detailed procedure of the isolation of stroma is described previously (Osteen *et al.*, 1989; Sugawara *et al.*, 1997; Khan *et al.*, 2005c).

The characteristics of the cultured stromal cells were determined by morphological and immunocytochemical studies. An aliquot of stromal cells was placed in a four-chamber slide (Nunc) for immunostaining and the rest was used for culture. After 24 h, the slides were washed in PBS, fixed with 4% paraformaldehyde for 10 min and rinsed with PBS. The slides then were incubated in 0.1% Triton X-100 for 5 min and were incubated for 3 h at 37°C as follows: against human cytokeratin monoclonal antibodies (mAb) (epithelial cell specific) at a dilution of 1:50 (MNF 116; Dako, Denmark), against human vimentin mAb (stromal cell specific) at a dilution of 1:20 (V9; Dako), against human von Willebrand factor mAb (endothelial cell specific) at a dilution of 1:50 (Dako) and against CD45 mAb (other leukocytes) at a 1:50 (Dako) dilution. The specificity of the immunocytochemical staining was confirmed by the deletion of the first antibody. Immunocytochemical staining was performed on at least three different isolated cells with similar results. The purity of stromal preparation was more than 95%, as judged by positive cellular staining for vimentin.

Treatment of M ϕ and stromal cells

The isolated peritoneal M ϕ were cultured in triplicate (10^5 cells/well) for 24 h to assess basal (constitutive) production of cytokines. To evaluate the stimulated (induced) secretion of cytokines, after initial culture with serum containing RPMI medium, M ϕ were serum starved for 24 h and then serum free M ϕ were cultured for another

24 h with different concentrations of highly purified recombinant human Hsp70 (1, 5, 10, 15 and 20 µg/ml) (low endotoxin, ESP-555, Stressgen, Victoria, Canada). A blocking experiment was performed with anti-TLR4 antibody (10 µg/ml) (HTA-125, HyCult Biotechnology) 20 min prior to the treatment with recombinant human Hsp70 (10 µg/ml) in order to examine any change in the secretion of cytokines and growth factors in culture media without washing the pre-incubated antibodies. After 24 h, the cultured media were collected in triplicate, pooled and frozen at -70°C until testing. Possible contamination of endotoxin with Hsp70 was examined by measuring endotoxin levels in the culture media by the limulus amoebocyte lysate test (Endotoxin-Single Test; Wako-Jun-Yaku Co. Ltd, Tokyo, Japan), pre-treatment of cells with polymyxin B (1 µg/ml, Sigma), an LPS antagonist and by heat treatment (65°C) of Hsp70-treated cells.

Next, to examine the direct effects of recombinant Hsp70 (1, 5 and 10 µg/ml) and LPS (10 ng/ml) derived from *Escherichia coli* (serotype 0111:b4; Sigma, St Louis, MO, USA) on the proliferation of endometrial stromal cells, 10⁴ cells/ml were plated in 96-well microtitre plate and treated with various doses of recombinant Hsp70 either alone or in combination with LPS (10 ng/ml) in serum free RPMI medium and incubated for another 24 h. The neutralizing effects of polymyxin B (1 µg/ml) and anti-TLR4 antibody (10 µg/ml) on stromal cell growth were examined.

The production of macromolecules in the culture media of basal and stimulated Mφ and cell proliferation assay were studied in six women with and without endometriosis (three each in proliferative phase and three in secretory phase).

Cytokine assays in the culture media of Mφ

The culture media of basal (non-treated) and stimulated (treated with Hsp70) Mφ were prospectively collected in triplicate and assays were performed retrospectively. The concentrations of hepatocyte growth factor (HGF), vascular endothelial cell growth factor (VEGF), interleukin (IL)-6 and tumor necrosis factor alpha (TNFα) in the culture media were measured in duplicate using a commercially available sandwich enzyme linked immunosorbent assay (ELISA) developed by R&D system in a blind fashion (Quantikine, R&D system, Minneapolis, MN, USA). The antibodies used in HGF, VEGF, IL-6 and TNFα determinations do not cross-react with other cytokines. The limits of detection were 40.0 pg/ml for HGF, 9.0 pg/ml for VEGF, 0.70 pg/ml for IL-6 and 4.4 pg/ml for TNFα. Both the intra- and inter-assay coefficients of variation were <10% for all these assays.

Immunolocalization of TLR4 in Mφ

In order to immunolocalize TLR4 in the CD68-immunoreactive isolated Mφ, we performed immunocytochemical staining of TLR4 using corresponding antibody (HTA-125, 1:50, Santa Cruz). The immunoreaction of TLR4 was examined in Mφ derived from women with or without endometriosis. The detailed procedure of immunocytochemistry was described previously (Fujishita *et al.*, 1997; Nisolle *et al.*, 1997; Khan *et al.*, 2005a,b). Non-immune mouse Ig G1 antibody (1:50) was used as a negative control.

Western blotting

Cultured cells in six-well plates were homogenized in the lysis buffer containing 50 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol, 50 mM dithiothreitol, and 0.1% bromophenol blue and were diluted to 1 mg total protein per milliliter. Plasma lysates and total cell lysates of Mφ and Ramos, a B-lymphoma cell line were resolved in 8% SDS-PAGE. The procedure in the preparation of plasma lysates and total cell lysates was described elsewhere

(Crawford *et al.*, 1982). Proteins were blotted onto a nitrocellulose membrane and incubated with a rabbit antibody to TLR4 (1:300) as a primary antibody and an anti-rabbit antibody (1:1000; Amersham Pharmacia Biotech, UK) as a secondary antibody. Immune complexes were visualized by the use of an enhanced chemiluminescence western blotting system (Amersham Pharmacia Biotech).

Gene expression of TLR4 and HGF in Mφ

Ribonucleic acid (RNA) was extracted from the cultured Mφ in 60 mm petridish (Greiner) using the monophasic solution of 40% phenol and ISOGEN method (Molecular Research Center, Tokyo), according to the manufacturer's protocol.

The expression of TLR4 was analyzed in the Mφ derived from women with or without endometriosis. The presence of mRNA encoding TLR4 in the basal Mφ was determined using forward and reverse primers synthesized to anneal with cDNA for TLR4. Amplification of cDNA reaction mixture for TLR4 was done. The mRNA expressions of TLR4 were analyzed by RT-PCR and using the sense and anti-sense primers of TLR4 as described previously (Hirata *et al.*, 2005). PCR generated bands were cloned and found to match the published sequences for the expected products.

The mRNA expression of HGF in response to Hsp70 and anti-TLR4 antibody was also examined by standard RT-PCR and using sense and anti-sense primers as described previously (Khan *et al.*, 2005a,b,c). A scanner densitometer was used to determine the ratio of intensity of each band relative to β-actin that was used as an internal control. Autoradiographs were analyzed to quantify the differences in levels of transcripts between Hsp70-treated and non-treated samples derived from the control women and women with endometriosis. Values of each transcript after the treatment with Hsp70 were normalized to 1. Densitometric analysis of gel bands was performed using the National Institutes of Health image analysis program.

Real-time quantitative PCR was performed as reported previously (Koga *et al.*, 2000). To assess TLR4 and HGF mRNA expression, real-time quantitative PCR and data analysis were performed using Light Cycler (Roche Diagnostics GmbH, Mannheim, Germany). Expression of TLR4 and HGF mRNA was normalized to RNA loading for each sample using β-actin mRNA as an internal control. The primers for TLR4, HGF and β-actin were the same as those used for standard PCR. PCR conditions were as follows: for TLR4, 40 cycles at 95°C for 10 s, 64°C for 10 s and 72°C for 12 s; for HGF, 40 cycles at 95°C for 10 s, 64°C for 10 s and 72°C for 12 s; for β-actin, 30 cycles at 95°C for 10 s, 60°C for 10 s and 72°C for 10 s. All PCR conditions were followed by the melting curve analysis.

Each PCR product was purified with a QIAEX II gel extraction kit (QIAGEN), and their identities were confirmed using an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster city, CA, USA).

Cell proliferation assays

5-Bromo-2-deoxyuridine (BrdU) can be used to measure cell proliferation by quantifying BrdU incorporated into the newly synthesized DNA of replicating cells (Takagi, 1993; Khan *et al.*, 2005a,b). The incorporated BrdU can be detected by a quantitative cellular enzyme immunoassay (Biotrak, Amersham Pharmacia Biotech Ltd, UK) using monoclonal antibodies directed against BrdU. It offers a non-radioactive alternative to the [³H]-thymidine-based cell proliferation and carries equal sensitivity and specificity (Takagi, 1993). The detailed procedure of BrdU incorporation assay was described previously (Khan *et al.*, 2005a,b,c). We examined the proliferation of endometrial stromal cells in response to Hsp70, LPS, polymyxin B and anti-TLR4 antibody and the differences in cell proliferation were expressed as the percentage of controls. The absorbance values

correlated directly with the amount of DNA synthesis and thereby with the number of proliferating cells in culture.

Endogenous Hsp70 assays in tissue extracts

A fraction of biopsy specimen from eutopic endometria of women with and without endometriosis and from different peritoneal lesions of women with endometriosis was homogenized in homogenizing buffer using a Polytron homogenizer (Kinematics, Luzern, Switzerland) and according to the procedure described previously (Miura *et al.*, 2006). The respective tissue suspension was centrifuged at 1500 rpm for 5 min to obtain the supernatant and stored at -80°C for the subsequent measurement of endogenous human Hsp70.

The tissue concentrations of human Hsp70 in the homogenized supernatant were measured in duplicate using a commercially available sandwich enzyme-linked immunosorbent assay (StressXpressTM, EKS-700, Stressgen, Victoria, Canada) according to the manufacturer's instructions. The protein concentration of samples was measured by the method of Bradford (1976) to standardize Hsp70 levels.

The antibodies used in Hsp70 determination do not cross-react with other cytokines. The sensitivity of this assay kit has been determined to be 200 pg/ml. Both the intra- and inter-assay coefficients of variation were $<10\%$ for this assay. The tissue concentration of Hsp70 was expressed as nanogram per microgram protein.

Statistical analysis

The clinical characteristics of the subjects were evaluated by one-way analysis of variance. The data are expressed as either mean \pm SEM or mean \pm SD. The concentrations of the studied cytokines were not distributed normally and the data were analyzed using non-parametric test. The differences between endometriosis and non-endometriosis, red lesions and other peritoneal lesions, Hsp70- or LPS-treated and non-treated groups were compared using Mann-Whitney *U*-test or Student's *t*-test. For comparison among three or more groups, the Kruskal-Wallis test was used to determine the difference among the groups. $P < 0.05$ was considered statistically significant.

Results

There were no significant differences in clinical characteristics between women with and without endometriosis (data not shown). As an initial study, we also examined five women with endometriosis but without infertility. We did not find any differences in cytokine profile or cell growth in response to Hsp70 in these five women compared with the women with endometriosis and infertility. Therefore, we represented our data only in women with infertility.

Expression of TLR4 in M ϕ

We detected both protein and gene expression of TLR4 in M ϕ , endometrial stromal cells and epithelial cells and also in eutopic and ectopic endometria derived from women with and without endometriosis. TLR4 was immunolocalized in CD68-positive M ϕ (Fig. 1A), vimentin-positive stromal cells and cytokeratin-positive epithelial cells. A parallel expression of TLR4 was also found in the glandular epithelial cells and stromal cells derived from both eutopic and ectopic endometria of women with and without endometriosis (data not shown). A 78 kDa molecular size of TLR4 was also visualized by western blot analysis in M ϕ and this was prominent in total cell lysates (Lane 3 for Ramos cells and Lane 4 for M ϕ) (Fig. 1B). This

was confirmed at the mRNA levels (406 bp) in basal M ϕ derived from the PF of women with and without endometriosis (Fig. 1C). The amount of TLR4 mRNA was dose-dependently increased in basal M ϕ with a maximum amount found at 24–48 h of incubated cells. Although an apparent increase in the amount of TLR4 mRNA was found in basal M ϕ derived from women with endometriosis, there was no significant difference in TLR4 expression between women with and without endometriosis (Table I). The protein and gene expression of TLR4 in endometrial cells were reported elsewhere (Young *et al.*, 2004; Hirata *et al.*, 2005; Khan *et al.*, 2005d,e, 2007).

TLR4-mediated production of different cytokines by human Hsp70-treated M ϕ

In our initial time-dependent and dose-dependent study, we found a maximum increase in the levels of different

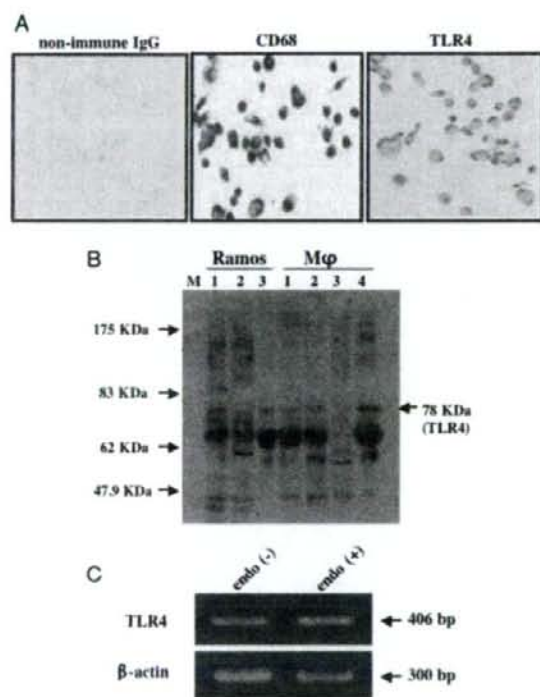


Figure 1: TLR4 protein and gene expression in macrophages. (A) The immunolocalization of Toll-like receptor 4 (TLR4) in CD68-positive macrophages (M ϕ) derived from the peritoneal fluid of women with or without endometriosis. (B) Western-blot analysis showing a band of TLR4 of 78 kDa molecular size. A lysate of Ramos, a B-lymphoma cell line was used to show a control band positive for TLR4. For Ramos cells, Lane 1 indicates plasma lysate, Lane 2 indicates 50% diluted fraction of plasma lysate, Lane 3 indicates expression in total cell lysates (B). For M ϕ , Lane 1 indicates plasma lysate, Lanes 2 and 3 indicate 50 and 100% dilution of plasma lysate, Lane 4 indicates TLR4 expression in total cell lysates. We found more expression of TLR4 in total cell lysates and minimal expression in diluted fraction of plasma lysate. (C) TLR4 mRNA (406 bp) expression was detected by standard RT-PCR. Total RNA was extracted from cultured M ϕ derived from three women each with endometriosis (endo+) and without endometriosis (endo-).

Table I. Time-dependent study of TLR4 relative gene levels in basal peritoneal macrophages (M ϕ).

	Incubation time (h)				
	0	6	12	24	48
Endometriosis (-)	1	0.48 \pm 0.12	0.55 \pm 0.11	1.12 \pm 0.13	1.42 \pm 0.15
Endometriosis (+)	1	0.67 \pm 0.15	0.87 \pm 0.16	1.67 \pm 0.22	1.75 \pm 0.22

Basal (non-treated) peritoneal M ϕ derived from women with and without endometriosis were cultured for different incubation time. The expression of TLR4 mRNA was determined by real-time quantitative PCR using LightCycler and was expressed as fold changes in relative gene levels. The values shown are fold of control at initial incubation period (0 h). Normalization was performed by respective expression level of beta-actin gene. All values express mean \pm SEM of three independent experiments. The relative gene level of TLR4 was time-dependently increased with a maximum level found at 24–48 h. No significant difference in TLR4 gene level was observed between women with and without endometriosis.

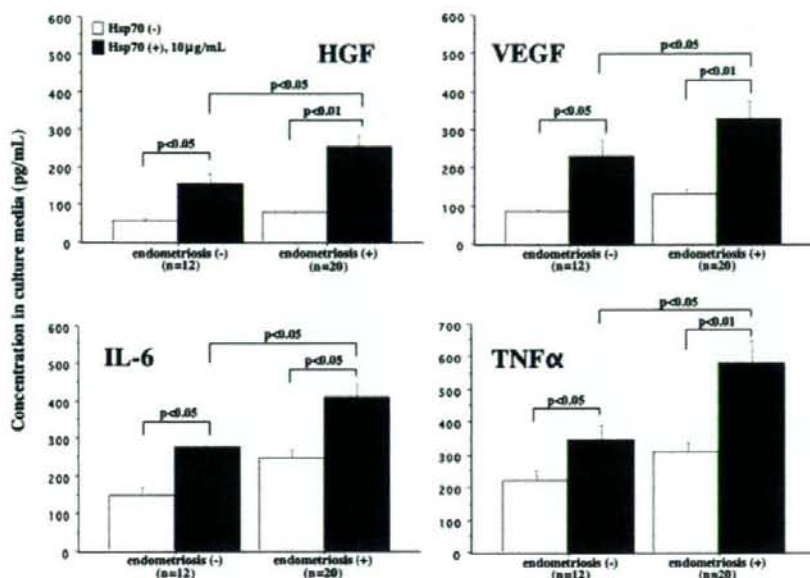


Figure 2: Production of different macromolecules by Hsp70-treated (black bar) and non-treated (white bar) M ϕ derived from the peritoneal fluid of women with and without endometriosis.

The levels of HGF, VEGF, IL-6 and TNF α in the culture media of M ϕ (10^5 cells/well) were significantly higher in the treated group comparing to non-treated group (either $P < 0.05$ or < 0.01), and were also significantly higher in women with endometriosis than in women without endometriosis ($P < 0.05$ for each). All these data are expressed as mean \pm SEM of three separate experiments for each group and are normalized for same number of cells.

macromolecules and cell growth at 24–48 h and in response to 10–15 μ g/ml of Hsp70. Therefore, we obtained all current experimental data in response to 10 μ g/ml of Hsp70 with a treatment duration of 24 h. We found that the concentrations of HGF, VEGF, IL-6 and TNF α were significantly higher in the culture media of Hsp70-treated M ϕ than that in non-treated M ϕ ($P < 0.01$ or < 0.05 for each, Fig. 2). The levels of these cytokines and growth factors were also markedly higher in M ϕ derived from women with endometriosis when compared with control women without endometriosis ($P < 0.05$ for each, Fig. 2). When we pre-treated M ϕ with antibody against TLR4, then again treated them with Hsp70, the levels of all these cytokines and growth factors were significantly decreased in comparison with cells without antibody ($P < 0.05$, for each of HGF, VEGF, IL-6 and TNF α) (Fig. 3). This effect was observed in M ϕ derived from women with endometriosis but not from control women. No difference in the

production of these macromolecules was observed between M ϕ collected during the proliferative phase and the secretory phase (data not shown).

TLR4-mediated gene expression of HGF by human Hsp70

We found that gene expression of HGF in response to Hsp70 is stronger in M ϕ derived from women with endometriosis than from women without endometriosis (Fig. 4A). Although a dose-dependent increase in gene expression of HGF (505 bp) was observed in both these two groups of women, a significant and a 2-fold increase in mRNA expression of HGF was found in women with endometriosis ($P < 0.05$) compared with women without endometriosis in response to Hsp70 (Fig. 4B). When we pre-treated M ϕ with antibody against TLR4, then again treated them with Hsp70, HGF mRNA expression level was significantly decreased in comparison with cells without antibody ($P < 0.05$, Fig. 4B). When we

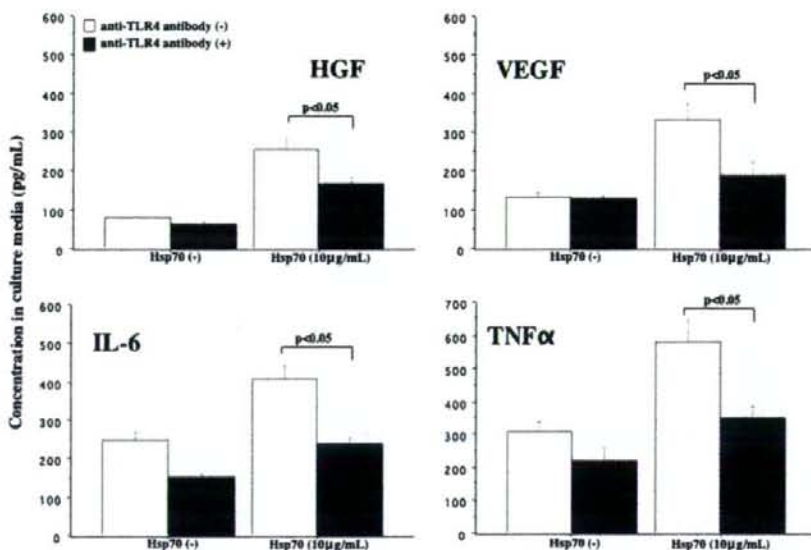


Figure 3: Neutralizing effect of anti-TLR4 antibody on the levels of HGF, VEGF, IL-6 and TNFα in the culture media of Mφ (10^5 cells/well) derived from the peritoneal fluid of women with endometriosis.

Mφ were pre-treated with anti-TLR4 antibody (10 μg/ml) (black bar) and without antibody (white bar) for 20 min and then further treated with and without Hsp70 for a period of 24 h. Pre-treatment of cells with anti-TLR4 antibody was able to significantly decrease all these macromolecules when compared with non-pre-treatment group ($P < 0.05$ for each). All these data are expressed as mean \pm SEM of three separate experiments for each group and were normalized with same number of cells.

examined the difference in the amount of HGF mRNA in response to Hsp70 and anti-TLR4 antibody by RT-PCR, results in Mφ were almost parallel to those obtained by standard RT-PCR (Table II). This indicates that the stimulating effect of Hsp70 on the production of HGF and other macromolecules is mediated by TLR4.

Exclusion of endotoxin contamination of Hsp70-treated cells

In order to exclude the possible contamination by endotoxin of Hsp70-treated cells, both Mφ and endometrial stroma, we repeatedly measured endotoxin level in the culture media. We could not detect any endotoxin in the culture media of Hsp70-treated Mφ or Hsp70-treated stromal cells. Pre-treatment of Mφ with polymyxin B (1 μg/ml) failed to decrease the levels of any of these macromolecules in the culture media of Hsp70-treated cells (data not shown). Since LPS is heat stable and Hsp70 is heat labile (Wallin *et al.*, 2002), we further excluded endotoxin contamination by heat treatment (65°C) of Hsp70-treated cells for 20 min. We could not detect any cytokine or growth factor in the culture media of Hsp70-treated cells, possibly due to degradation of Hsp70 after heat treatment.

Proliferation of stromal cells by Hsp70 and LPS

A BrdU incorporation study indicated that stromal cells derived from eutopic endometria of women with endometriosis proliferated dose-dependently and significantly in response to Hsp70 ($P < 0.05$ at 5 and 10 μg/ml versus non-treated cells, Fig. 5A). No significant difference was observed between treated and non-treated cells derived from women without endometriosis.

We also found that the individual treatment with Hsp70 (10 μg/ml) or LPS (10 ng/ml) was able to significantly stimulate proliferation of stromal cells derived from eutopic endometria of women with endometriosis (1.5- to 1.7-fold increase) compared with non-treated cells ($P < 0.05$ for each, Fig. 5B). A synergistic effect in cell proliferation was observed between Hsp70 and LPS. In fact, combined treatment of stromal cells with Hsp70 and LPS further increased BrdU incorporation when compared with cells treated with Hsp70 alone ($P < 0.05$, Fig. 5B). In order to confirm the cellular specificity of LPS, we treated cells with polymyxin B (1 μg/ml) and an LPS antagonist. We found that polymyxin B significantly abrogated LPS-promoted cell proliferation ($P < 0.05$) but failed to decrease combined Hsp70- and LPS-promoted proliferation of stromal cells (Fig. 5B). However, pre-treatment of stromal cells with anti-TLR4 antibody (10 μg/ml) was able to significantly decrease the combined Hsp70- and LPS-promoted proliferation of stromal cells ($P < 0.05$, Fig. 5B). These results further indicate that both Hsp70 and LPS have the capacity to directly stimulate stromal cell proliferation and this growth promoting effect is mediated by TLR4.

Tissue levels of endogenous Hsp70 in eutopic and ectopic endometria

In order to examine the *in vivo* variation of stress reaction at the tissue level, we measured levels of endogenous Hsp70 in the eutopic endometria of women with and without endometriosis and in different peritoneal lesions as shown in Fig. 6. The tissue concentrations of Hsp70 were significantly higher in the homogenized samples of eutopic endometria derived from

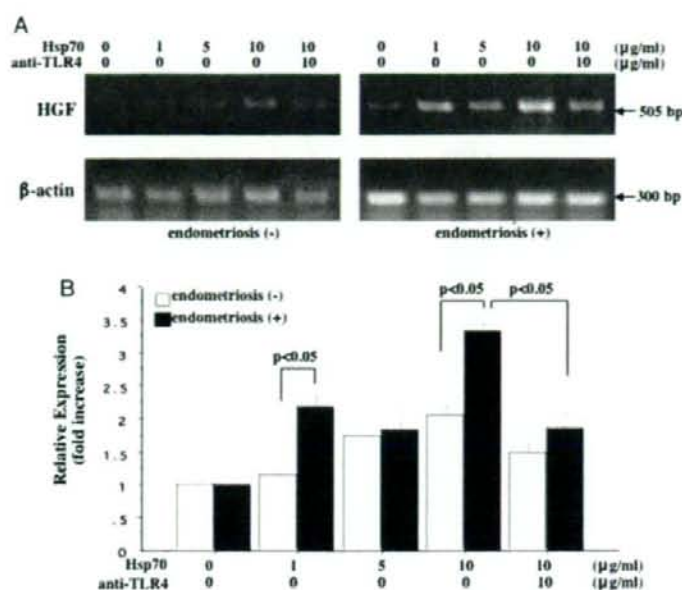


Figure 4: HGF gene expression in macrophages by human Hsp70.

(A) The effect of a variable concentration of Hsp70 (0–10 µg/ml) and anti-TLR4 antibody (10 µg/ml) on the mRNA expression of HGF in peritoneal Mφ derived from women with or without endometriosis was studied by standard RT-PCR. (B) The individual mRNA band (505 bp) of HGF was normalized with the corresponding band of internal control (β-actin) and is represented by the fold increase of their corresponding control (without treatment with Hsp70). Values of each transcript after single treatment with Hsp70 or pre-treatment with anti-TLR4 antibody (10 µg/ml) were normalized to 1 (dose 0). For HGF, a significance of $P < 0.05$ was found at the dose of 1 and 10 µg/ml of Hsp70 (endometriosis versus non-endometriosis) and $P < 0.05$ was found when compared with anti-TLR4 antibody non-treated Mφ. The results are expressed as mean ± SEM of three different experiments derived from three separate patients.

Table II. Effects of Hsp70 and anti-TLR4 antibody on relative gene level of HGF in peritoneal macrophages (Mφ).

Hsp70 (µg/ml)	0	1	5	10	10
anti-TLR4 antibody (µg/ml)	0	0	0	0	10
Endometriosis (-)	1	1.26 ± 0.19	1.57 ± 0.31	2.24 ± 0.28	1.81 ± 0.18
Endometriosis (+)	1	2.38 ± 0.37	2.89 ± 0.46	3.91 ± 0.21*	1.96 ± 0.34*

Peritoneal Mφ were treated with or without Hsp70 for a period of 24 h. Mφ were pretreated with anti-TLR4 antibody and incubated for another 24 h. The expression of HGF mRNA was determined by real-time quantitative PCR using LightCycler and was expressed as fold changes in relative gene levels. The values shown are fold of control without any treatment. Normalization was performed by respective expression level of beta-actin gene. All values express mean ± SEM of three independent experiments.

The relative gene level of HGF was dose-dependently increased and this was significantly higher in Mφ derived from women with endometriosis than that in non-endometriosis. * $P < 0.05$ versus without anti-TLR4 antibody.

women with endometriosis than in similar tissues derived from control women ($P < 0.05$, Fig. 6A). No significant difference was observed in tissue levels of Hsp70 between endometriosis of Stages I–II and III–IV (data not shown). Although an apparent increase in tissue levels of Hsp70 was found in the samples derived from women in the secretory phase, we did not find any significant difference in Hsp70 levels in endometrial samples between the secretory phase and the proliferative phase (data not shown).

When we analyzed endogenous Hsp70 levels in samples according to color appearance of peritoneal lesions in women with pelvic endometriosis, we found that the tissue levels were highest in red lesions, intermediate in black lesions and lowest in white peritoneal lesions ($P < 0.05$ by Kruskal–Wallis test for red lesions, Fig. 6B). No difference in tissue levels of Hsp70 was found between the samples of black

lesions and white lesions. When total red lesions were subdivided into blood-filled opaque red lesions and non-opaque transparent or translucent red lesions, as we described previously (Khan *et al.*, 2004b), opaque red lesions showed significantly higher levels of Hsp70 at the tissue level when compared with either non-opaque red lesions, black lesions or white peritoneal lesions ($P < 0.01$ for each, Fig. 6B). No difference in tissue levels of Hsp70 was found among less active peritoneal lesions, such as non-opaque red lesions, black lesions and white peritoneal lesions.

Discussion

In our current study, we demonstrated that pelvic endometriosis induces stress reaction in the pelvic environment in addition to inducing pelvic inflammation. This was confirmed by the

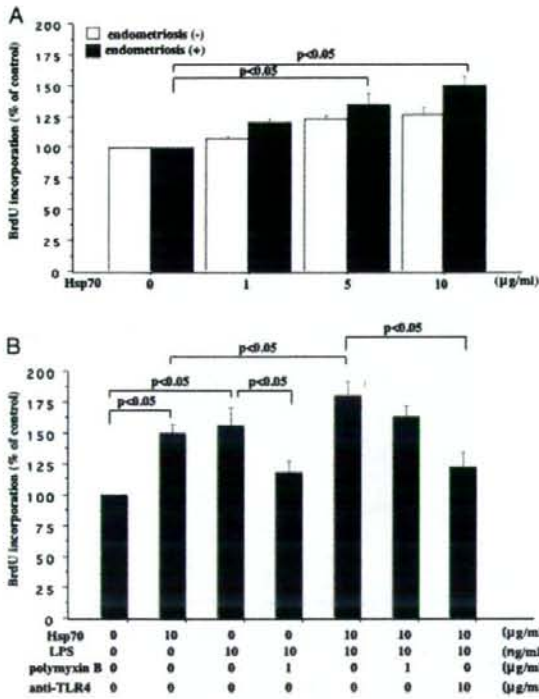


Figure 5: Single and combined effect of exogenous Hsp70 and LPS on the proliferation of stromal cells derived from the eutopic endometria of women with endometriosis (black bar) and without endometriosis (white bar) as measured by 5-bromo-2-deoxyuridine incorporation. Different concentrations of recombinant human Hsp70 (0, 1, 5, 10 µg/ml) were applied to stromal cells as shown in (A). The single and combined treatment of stromal cells with Hsp70 (10 µg/ml), LPS (10 ng/ml) and pre-treatment of these cells with either polyxymyxin B (1 µg/ml) or anti-TLR4 antibody (10 µg/ml) are shown in (B). The results are represented as percentage of control (without any treatment) expressed as mean ± SEM of three different experiments derived from three separate patients. **A.** $P < 0.05$ versus non-treated cells; **B.** $P < 0.05$ (Hsp70 versus control); $P < 0.05$ (LPS versus control); $P < 0.05$ (LPS alone versus LPS + polyxymyxin B); $P < 0.05$ (combined Hsp70 + LPS versus control); $P < 0.05$ (anti-TLR4 pre-treated cells versus without anti-TLR4 pre-treated cells).

release of a variable amount of endogenous Hsp70 by the different peritoneal lesions and eutopic endometria of women with endometriosis. We also demonstrated that locally produced Hsp70 might be responsible for TLR4-mediated induction of inflammatory reaction and direct promotion in the growth of endometriosis. Although polyxymyxin B, a potent LPS antagonist, is able to suppress LPS-mediated growth of endometrial cells derived from women with endometriosis as reported previously (Hirata *et al.*, 2005; Khan *et al.*, 2007), in our current study, polyxymyxin B was unable to suppress combined LPS- and Hsp70-mediated growth of endometriosis. In contrast, the growth promoting effect of combined LPS and Hsp70 was significantly suppressed when the biological function of TLR4 was blocked with anti-TLR4 antibody. Our current findings indicated that LPS- and Hsp70-mediated inflammatory reaction and growth of endometriosis may be mediated by TLR4 in the pelvic environment.

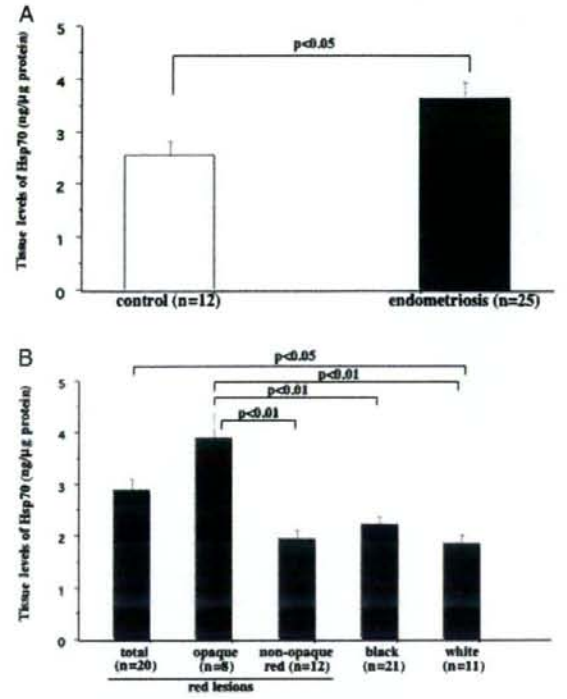


Figure 6: Tissue levels of Hsp70 in the eutopic and ectopic endometria derived from women with and without endometriosis. The concentration of Hsp70 was measured in the supernatant of tissue homogenates derived from the eutopic endometria (A) and different peritoneal lesions (B). The results are expressed as mean ± SEM. The tissue levels of Hsp70 were significantly higher in the eutopic endometria of women with endometriosis when compared with that in the similar tissues derived from control women ($P < 0.05$, A). When we examined tissue levels of Hsp70 in different peritoneal lesions of women with endometriosis (B), we found that blood-filled opaque red lesions contained significantly higher amount of Hsp70 than that of either non-opaque red lesions ($P < 0.01$) or black lesions ($P < 0.01$) or white lesions ($P < 0.01$). The tissue level of Hsp70 was the highest ($P < 0.05$) in total red lesions compared to black or white lesions.

TLR4-mediated production of different cytokines and growth factors and endometrial cell proliferation in response to LPS has been demonstrated previously (Hirata *et al.*, 2005; Khan *et al.*, 2005d,e, 2007). All of these studies were done in *in vitro* culture system. When we consider the internal pelvic environment, besides LPS, there are a number of other exogenous and endogenous ligands for TLR4 (Kiechl *et al.*, 2002; Akira and Takeda, 2004). TLR4 is an essential receptor for bacterial endotoxin or LPS recognition. In addition to LPS, as a potential endogenous ligand, Hsp70 can also transmit signal through TLR4 (Wallin *et al.*, 2002; Triantafyllou and Triantafyllou, 2004). Therefore, we presumed that growth of endometriosis may be regulated by endogenous Hsp70 or LPS either alone or in combination after their binding with TLR4. The expression level of TLR4 was reported to be higher in the samples derived from the secretory phase of menstrual cycle (Fazeli *et al.*, 2005; Aflatoonian *et al.*, 2007), however, we did not find any significant difference in

TLR4 expression levels between proliferative phase and secretory phase samples or between women with and without endometriosis in our current study. This could be due to the small number of samples we used in the current study.

As a component of the innate immune system, we found that M ϕ derived from the PF of women with and without endometriosis equally expressed TLR4 both at the protein and gene level. When we measured the secretion levels of HGF, VEGF, IL-6 and TNF α in the culture media of Hsp70-treated M ϕ , we found that the production of all these macromolecules was significantly higher in treated cells when compared with non-treated cells or in M ϕ derived from women with endometriosis when compared with women without endometriosis. Again, direct stimulation with Hsp70 was able to significantly enhance the proliferation of endometrial stromal cells derived from women with endometriosis compared with similar cells derived from control women. All these pro-inflammatory response and cell promoting effects of Hsp70 were mediated by TLR4. In fact, we found a similar increase in the amount of HGF mRNA in M ϕ in response to Hsp70 and abrogation of cytokine secretion, HGF mRNA levels and cell proliferation after pretreatment of cells with anti-TLR4 antibody. Our current findings provide further evidence that an internal stress reaction in the pelvic environment could be responsible for pelvic inflammation and growth of endometriosis in addition to estrogen and other primary or secondary inflammatory mediators.

We learned from our present study that blocking of TLR4 could be more effective in reducing pro-inflammatory response and growth of pelvic endometriosis, because there are other endogenous and exogenous ligands for TLR4 in addition to LPS (Kiechl *et al.*, 2002). The possible contamination by endotoxin in the study of Hsp70-treated cells during bio-culture procedure was a matter of concern in different reports (Byrd *et al.*, 1999; Triantafilou *et al.*, 2001; Wallin *et al.*, 2002; Triantafilou and Triantafilou, 2004). However, we carefully excluded the possible contamination of Hsp70-treated cells with endotoxin by our serial exclusion experiments.

Endogenous Hsps including Hsp70 can be produced in response to environmental stimuli (heat shock, ultraviolet radiation and heavy metals), pathological stimuli (viral, bacterial, parasitic infection, inflammation, malignancy or autoimmunity) and physiological stimuli (different physical or chemical stress) (Asea *et al.*, 2000, 2002). Besides inflammation, pelvic endometriosis may induce a variable degree of physical stress (cell to cell contact, cell proliferation, cell differentiation or tissue invasion) or chemical stress (receptor–ligand interaction) in the pelvic environment. In order to examine the degree of stress reaction in the pelvic environment, we measured tissue levels of endogenous Hsp70 in the eutopic endometria and different peritoneal lesions of women with endometriosis. We found that endogenous stress reaction in eutopic endometria as measured by tissue levels of Hsp70 was significantly higher in women with endometriosis when compared with control women. Although a tendency of higher tissue levels of Hsp70 was observed in early endometriosis (Stages I–II) and in the secretory phase, there was no significant difference in Hsp70 levels between r-ASRM Stages I–II and III–IV or between proliferative and secretory phases of the menstrual cycle.

When we analyzed tissue levels of Hsp70 according to color appearance of endometriotic lesions, we found the highest levels in blood-filled opaque red lesions compared with other peritoneal lesions. This could be due to a higher stress reaction caused by increased mitogenic, angiogenic and tissue invasion properties of opaque red lesions as we reported previously (Khan *et al.*, 2003, 2004b). In fact, both opaque and non-opaque red lesions are included in the same groups of red lesions according to the morphological classification of r-ASRM (ASRM, 1997). These results further indicated that this subgroup of red lesions displayed strong stress reaction when compared with other peritoneal lesions in the pelvic environment.

Finally, we conclude that women with endometriosis harboring peritoneal lesions of different color appearances suffer different *in vivo* stress reaction. Among them, a prominent stress reaction was observed in blood-filled opaque red peritoneal lesions. We suggest that human Hsp70 also induces pelvic inflammation and may regulate TLR4-mediated growth of endometriosis. A variation in pelvic inflammatory reaction and stress reaction may function together to regulate the growth of pelvic endometriosis. Our current findings may provide a clue in targeting TLR4 as a new therapeutic strategy in women with endometriosis. Current ongoing studies from our laboratory regarding relationships between stress reaction and inflammation or between stress reaction and angiogenic response in the pelvic environment may provide further evidence for the importance of stress protein in women with different endocrine diseases.

Acknowledgements

We thank Miss Kazumi Hayashida and Miss Kyoko Ishida, Department of Obstetrics and Gynecology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan, for their excellent technical assistance.

Funding

This work was supported by Grants-in-Aid for Scientific Research (Grant No. 16591671 and 18591837) from the Ministry of Education, Sports, Culture, Science and Technology of Japan (to KN Khan).

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Submitted on December 24, 2007; resubmitted on April 16, 2008; accepted on April 25, 2008

循環器症候群(第2版)

IV

—その他の循環器疾患を含めて—

- XII. 染色体構造異常・奇形症候群にみられる
心・血管・血圧異常

Wolf-Hirschhorn 症候群と
猫なき症候群

三浦清徳 新川詔夫

XII 染色体構造異常・奇形症候群にみられる心・血管・血圧異常

Wolf-Hirschhorn 症候群と猫なき症候群

Wolf-Hirschhorn syndrome and cat cry syndrome

Key words : Wolf-Hirschhorn 症候群, 猫なき症候群, 隣接遺伝子症候群, マイクロアレイ技術

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はじめに

最近のゲノム解析技術の進歩により, Wolf-Hirschhorn 症候群と猫なき症候群は隣接遺伝子症候群(contiguous gene syndrome)の範疇に分類される。患者の多くが合併する先天性心奇形は生命予後と関連する。本稿では, 各々の疾患について循環器異常に関する知見を交えて解説する。

Wolf-Hirschhorn 症候群(WHS)

1. 概念・定義—同義語—

4 番染色体の短腕部分欠失[del(4p)]により, 重度の成長障害, 精神遅滞, 小頭, 特異顔貌, 閉鎖障害奇形(口唇・口蓋裂, 心房中隔欠損, 心室中隔欠損など)を特徴とする染色体異常症である¹⁻⁶⁾。

同義語 : 4p-症候群(4p- syndrome)。

2. 病 因

del(4p)に起因する。症例により 4p の欠失範囲は様々だが, 責任領域は WHSCR(Wolf-Hirschhorn syndrome critical region)と WHSCR2 の 2 領域が同定され⁷⁾, いずれも 4 番染色体短腕テロメア側から 1.27-2.2 Mb の 4p16.3 に存在する⁸⁾。WHSCR には *WHSC1*(Wolf-Hirschhorn syndrome candidate 1)および *WHSC2* が存在し, WHSCR2 には *LETM1*(leucine zipper-EF-hand containing transmembrane protein 1), *FGFR3*

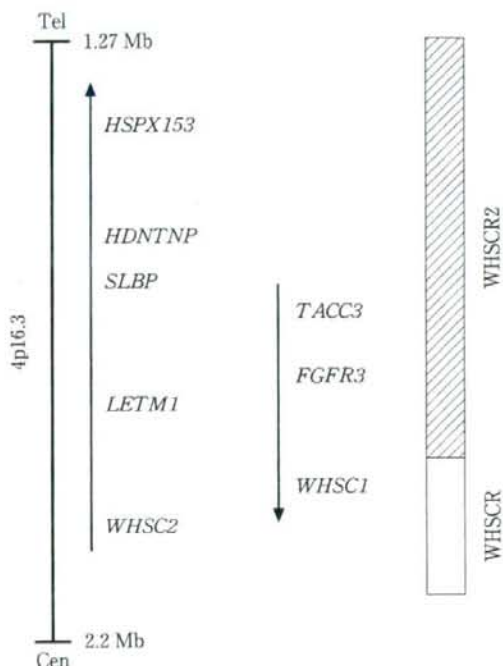
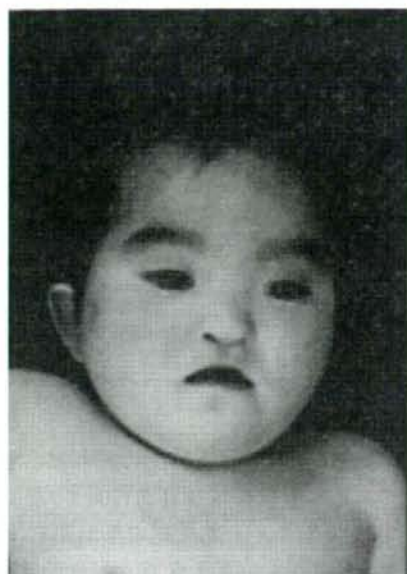


図1 Wolf-Hirschhorn 症候群の責任領域

責任領域は 4 番染色体短腕末端(pter)側から 1.27-2.2 Mb の領域(4p16.3)に存在し, WHSCR および WHSCR2 の 2 つの領域が同定されている。→は転写方向を示す。

(fibroblast growth factor receptor 3), *TACC3*(transforming acidic coiled-coil containing protein 3), *SLBP*(stem-loop-binding protein), *HDNTNP*(human ortholog of dorsal neural tube nuclear protein) および *HSPX153* が含まれている(図1)。いずれも単独の欠失では WHS の症状を呈しないため, WHS は欠失領域に存在す

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a.



b.

図2 Wolf-Hirschhorn 症候群患者の顔貌(a)と古代ギリシャ兵士の兜(b)

る複数の遺伝子が病態に関与する隣接遺伝子症候群と考えられる⁹⁾。大多数は新しく生じた腕内欠失だが、親の転座や環状染色体由来の欠失も多く認められる⁹⁾。

3. 病 態

罹病率は出生1/50,000人である⁸⁾。しかし、最近のマイクロアレイ技術の進歩と普及により、その検出頻度は上昇しているため、実際の罹病率は報告よりも高いと推定される¹⁰⁾。性比は男性1:女性2で女性に多い。欠失の範囲と表現型の重症度との間には相関関係は認められない¹¹⁾。一方、新生欠失例では欠失の大きさと死亡率との間に相関がある¹⁰⁾。小頭、眼間開離、眉間部突出、短い人中、魚様の口、小顎症、口唇・口蓋裂などから古代ギリシャ兵のような顔貌を呈する(図2)。発育障害は著明で、子宮内胎児発育遅延を呈し、低出生体重児として出生する。その後の発育も遅れ、骨年齢は遅延する。精神遅滞の程度(IQ<20)も重度である。心奇形(後述)、脳梁欠損、腎奇形、横隔膜ヘルニア、尿道下裂、難治性けいれんなども認める。

4. 循環器異常

50%以上の症例で心奇形を合併し⁹⁾、大血管異常や総肺静脈還流異常、心房中隔欠損、心室中隔欠損などの複合奇形を伴うことが多い。

5. 診 断

染色体検査もしくはマイクロアレイCGH(comparative genomic hybridization)解析により、4番染色体短腕の部分欠失を検出する。猫なき症候群との鑑別は臨床症状から容易である。

6. 治療と予後

心奇形などの重度な先天奇形を伴えば予後は不良であり、患者の21%は出生後2年以内に死亡する¹⁰⁾。

猫なき症候群(cat cry syndrome)

1. 概念・定義—同義語—

5番染色体の短腕部分欠失[del(5p)]により、乳児期における子猫のような甲高い泣き声、特

異な顔貌、精神遅滞を特徴とする染色体異常症である^{12,13)}。

同義語：5p-症候群(5p- syndrome), cri-du-chat syndrome.

2. 病 因

del(5p)に起因する。欠失部位は様々であるが⁵⁾、5p15-5p14の欠失は全症例に認められる。新生腕内欠失が多いが⁶⁾、約12%は親の相互転座由来の欠失である。まれに環状染色体由来の欠失やモザイクを伴う症例も知られている。複数の遺伝子を包括的に欠失する隣接遺伝子症候群であり、甲高い泣き声は5p15.31、言語遅滞は5p15.32-5p15.33、および特異顔貌は5p15.2-5p15.31に責任遺伝子が存在すると推定される¹⁴⁾。精神遅滞の責任領域は近位端にかけてMR-I、MR-IIおよびMR-IIIの3領域に分けられ、精神遅滞の程度は欠失部位が近位端に行くにつれて軽症である¹⁴⁾。また、5p15.33に位置する*hTERT*(telomerase reverse transcriptase)遺伝子の欠失によるハプロ不全が、本症候群の症状における多様性と関連している可能性も指摘されている¹⁵⁾。

3. 病 態

罹病率は出生1/20,000-1/50,000人である⁹⁾。しかし、最近のマイクロアレイ技術の進歩と普及により、欠失検出率は高まり、実際の罹病率は報告よりも高いと予想される。重度な精神遅滞児(IQ<20)の約1%が本疾患である¹³⁾。性比は男性5:女性7で女性に多い。欠失の程度と表現型の重症度との間に関連が示唆されている¹⁶⁾。

出生時から子猫のような甲高い泣き声を認めるが(100%)、幼児期以降では消失する(ただし声は甲高い)。乳児期には小頭(-3SD)、眼間開離を伴う円形顔貌、小顎、内眼角贅皮、耳介低位、鞍鼻などの特異顔貌(80-90%)、および高口蓋を認める。しかし、年長者では顎が突出して細長くなり、眼間は正常あるいは短く変貌する。発育発達障害(100%)は著明で、IUGR・低

表1 猫なき症候群における先天性心疾患の分布(文献¹⁰⁾より改変)

先天性心疾患	猫なき症候群患者数 (n=41)	
動脈管開存症	18	(44%)
心室中隔欠損症	12	(30%)
心房中隔欠損症	4	(10%)
ファロー四徴症	3	(7%)
肺動脈狭窄症	1	(2%)
部分房室管症	1	(2%)
心房粗動	1	(2%)
異所性右鎖骨下動脈	1	(2%)

出生体重児として出生し、その後の体重増加も不良である。重度の精神遅滞(100%)を認め、IQは20-30である。乳児期の筋緊張低下は成長とともに筋緊張亢進となる。心奇形(後述)、口唇裂、口蓋裂、腎奇形、腹直筋離開、鼠径ヘルニア、中手骨・中足骨短縮などの異常も多い。

4. 循環器異常

心奇形は、本症の15-20%に合併する¹⁷⁾。その種類は様々だが、動脈管開存症の頻度は先天性心疾患を伴う一般集団と比較しても有意に高い(表1, 2)¹⁸⁾。このことが、他の染色体異常症に比べてやや低い新生児・乳児期死亡率の理由だと思われる。また、染色体異常のタイプと合併する心奇形の種類とを比較すると、孤発例は心室中隔欠損や心房中隔欠損などの中隔欠損症と関連し、不均衡型転座例はファロー四徴症や心内膜床欠損症などのより複雑な心血管奇形と関連しているという報告もある¹⁸⁾。

5. 診 断

染色体検査もしくはマイクロアレイCGH解析により、5番染色体短腕の部分欠失を検出する。

6. 治療と予後

約10%の症例は出生後1年以内に死亡するが、大多数は成人まで生存する。56歳までの生存例がある。